Upregulation of heme oxygenase-1 protected against brain damage induced by transient cerebral ischemia-reperfusion injury in rats

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Abstract. The aim of the present study was to identify the effect of heme oxygenase (HO)-1 gene on cerebral ischemia-reperfusion injury. Sprague-Dawley rats were divided randomly into four groups: Sham group, vehicle group, empty adenovirus vector (Ad) group and recombinant HO-1 adenovirus (Ad-HO-1) transfection group. Rats in the vehicle, Ad and Ad-HO-1 groups were respectively injected with saline, Ad or Ad-HO-1 for 3 days prior to cerebral ischemia-reperfusion injury. Subsequently, the middle cerebral artery occlusion method was used to establish the model of cerebral ischemia-reperfusion injury. Following the assessment of neurological function, rats were sacrificed, and the infarction volume and apoptotic index in rat brains were measured. Furthermore, the protein expression levels of HO-1 in brain tissues were detected using western blot analysis. Results indicated that the neurological score of the Ad-HO-1 group was significantly increased compared with the Ad or vehicle groups, respectively (P<0.001). The volume of cerebral infarction and the index score of neuronal apoptosis in the vehicle and Ad groups was significantly increased compared with the Ad-HO-1 group (P<0.01). The death of neuronal cells following cerebral ischemia-reperfusion injury reduced remarkably induced by over-expression of HO-1. These findings suggest a neuroprotective role of HO-1 against brain injury induced by transient cerebral ischemia-reperfusion injury.

Introduction

The research on ischemic preconditioning (IPC) has demonstrated that IPC-induced brain tolerance may be early or late, with the early phase ranging between min and h after the onset of IPC, and the late phase ranging between h and days after the onset of IPC (1). Furthermore, IPC activates several protective molecular pathways, including protein kinase C, mitogen-activated protein kinase and phosphatidylinositol 3-kinase at different time points (1). A previous study has indicated that, between 12 and 24 h after IPC, the expression of heat shock protein heme oxygenase (HO)-1 was activated in newborn rat brains, and upregulation of HO-1 expression remained for 7 days (2). A previous study also indicated that HO-1 was able to partially mediate the neuroprotective roles on IPC (3).

It is known that the rate-limiting enzyme HO is able to degrade heme *in vivo* and subsequently antagonize stress reactions, resulting in a protective effect against cell damage (4). The pro-oxidant heme may be subjected to oxidative degradation into the potent antioxidants carbon monoxide, biliverdin, and free iron by HO (5). Subsequently, biliverdin reductase is converted rapidly from biliverdin to bilirubin (4-6). There are two isoenzymes of HO, namely HO-1 and HO-2 (6). HO-2 gene knockout reduces oxidative stress in nerve cell damage (6). Conversely, HO-1 knockout mice are more sensitive to ischemia-reperfusion injury; however induced HO-1 overexpression may promote a protective effect (7). Furthermore, HO-1 upregulation may provide protection against damage induced by cold ischemia (7). These findings suggest that promoting the activity of HO-1, may have a potential cytoprotective effect against cell damage. In HO-1 transgenic rats, overexpression of HO-1 has been demonstrated to attenuate ischemic stroke damage induced by middle cerebral artery occlusion (MCAO) (8). It has been indicated that the activity of HO-1 with pharmacological stimulation may be a novel therapeutic target for the treatment of ischemic injury (8-10).

Gene transfer was used in the present study to investigate stroke by transducing genes into neurons (11). There are alternative approaches for expressing transgenes of interest in adult animals, and then investigating their effects on cerebral ischemia-reperfusion injury (11). One viral gene transfer method involves the use of recombinant adeno-associated viral vectors, which may deliver transgenes into neurons through a number of delivery pathways, including intramuscular (12), subcutaneous, intraneural (13), intrathecal (14,15) and intraspinal applications (16,17), direct injections into dorsal root ganglia (18), and application of virus accompanied with herpes simplex virus locality (19).

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The objectives of the present study included the following: i) Identification of the role of HO-1 gene in cerebral ischemia-reperfusion injury based on the transfection method; ii) confirmation of the high expression of the HO-1 gene and its anti-apoptotic effects; and iii) to illuminate the cellular or physiological effect of recombinant adenovirus bearing HO-1 (Ad-HO-1) against ischemia-reperfusion injury of the brain, which may have a protective role against stroke or neurological disorders associated with stroke.

Materials and methods

Adenovirus vector production. The adenovirus-bacterial recombination system (AdEasy) was supplied by Dr Chen Jing from Thomas Jefferson University (Philadelphia, PA, USA), including the expression vectors pGEM-3ZF (+) and pAdEasy-1, which were labeled with green fluorescent protein (GFP). The rat HO-1 gene was constructed into adenoviral vectors in the AdEasy Vector System as described previously (20). The spleens were taken from 6 3-month-old female Wistar rats (weight, 250±25 g) and stored at -80°C. The rats were maintained within a specific pathogen free facility with a constant humidity (55±5%) and temperature (22±2°C) in a 12 h light/dark cycle with free access to food and water. Total RNA was extracted from the rats spleen cells using an RNeasy Total RNA Isolation kit (Qiagen, Inc., Valencia, CA, USA). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed and the products were used for the gene amplification of HO-1, and subsequently cloned into pAdEasy-1 vectors. The RNA (2 μg per reaction) was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega Corporation, Madison, WI, USA) at 42°C for 70 min in the presence of 250 μM dNTPs, avian myeloblastosis reverse transcription 5X reaction buffer (250 mM Tris-HCl, 250 mM KCl, 50 mM MgCl$_2$, 50 mM DTT and 2.5 mM spermidine; all Promega Corporation) and 1 μM oligo-p (dT)15 primer (Roche Molecular Diagnostics, Pleasanton, CA, USA). PCR amplification reactions were performed in a total volume of 20 μl containing 1.5 U Taq DNA polymerase, 2 μl 10X PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl$_2$), 0.4 μl 10 mM dNTP (all Promega Corporation) and ~100 ng DNA template on a GeneAmp® PCR System 9700 (Roche Molecular Diagnostics). The thermocycling conditions were as follows: Predenaturing at 94°C for 5 min, followed by 32 cycles of denaturing at 94°C for 45 sec, annealing at the temperature appropriate for each pair of primers for 45 sec and extending at 72°C for 45 sec with a final extension at 72°C for 10 min. According to the published GeneBank sequence of rat HO-1 (NM_010442) (ncbi.nlm.nih.gov/nuccore/NM_010442.2?report=genbank), the upstream primer sequence was 5'-ATGGAGCGTCCACAGCTCGAC-3' and the downstream primer sequence was 5'-GGGCCA ACACTGCATTTACAT-3'. The HO-1 gene was synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Prior to use, assessments for viral concentration and titer of the pAdEasy-1 viral vectors were conducted. The concentration of virus [plaque-forming unit (pfu)/ml] was measured (21). The 50% tissue culture infection dosage (TCID50) method was used to measure virus titers. The cells were seeded into clear, flat-bottomed 96-well plates at 10^5 cells in 100 μl of DMEM containing 10% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1% L-glutamine, and 1% penicillin/streptomycin. The cells were added to all wells except column 10, which was left blank to separate the infected wells and the uninfected control wells. Prior to the assay an additional 80 μl of maintenance media with DMEM containing 2% FCS was added to all wells. A total of 20 μl neat or diluted virus was added to each well in the first column of the plate. The virus was then serially diluted (1:10, 20 μl into 180 μl) across the plate from columns 1 to 9 with pipette tips changed between each column. Column 10 was left empty, and columns 11 and 12 contained media only and served as controls for uninfected cells. The plates were then incubated at 37°C with 5% CO$_2$ for 6 days. On the 6th day, 3 μl (1.5%) Neutral Red solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to every well containing cells to aid visualization and the plates were returned to the incubator for an additional 24 h. On the 7th day postinoculation, the cells were fixed with 100 μl 10% formal-saline (prepared by diluting 37% formaldehyde with PBS, pH 7.4). Following fixation for a minimum of 30 min at room temperature, all liquid was removed from the plate and each well was examined for cytopathic effect. For each dilution, the number of wells that were positive for CPE was scored. A Reed and Muench calculation was then performed to determine the 50% infectious dose, which was then scaled up to give a count of the TCID50 per ml (21). The viral titers of Ad-GFP and Ad-HO-1 were 2.0×10^{11} pfu/ml and 2.5×10^{11} pfu/ml, respectively. Briefly, the vector plasmids and ViraPower Package mixture (containing the pLP1, pLP2 and pLP/VSVG plasmids) were cotransfected into 293T cells and cultured at 37°C. At 48 h post transfection the viruses were harvested and concentrated in PBS by ultracentrifugation (for 16 h at 9,000 x g at 4°C) to 1.25×10^9 transducing units/ml. The virus titer kit was purchased from MellGen Laboratories, Inc. (Surrey, BC, Canada) and used according to the manufacturer's protocol (21).

Animals and adenovirus infection. Based on the National Institutes of Health Guidelines on Use of Laboratory Animals, all experiments were conducted and approved by the Second Hospital of Shanxi Medical University Committee on Animal Care (Shanxi, China).

Male Sprague-Dawley (SD) rats (n=48; weight, 240-280 g; age, 3-6 months) were purchased from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). The rats were maintained in a specific pathogen free facility with a constant humidity (55±5%) and temperature (22±2°C) and a 12 h light/dark cycle with free access to food and water. Rats were randomly divided into 4 groups (n=6/group): Sham group, vehicle group, empty adenovirus vector (Ad) group and Ad-HO-1 transfection group. Rats in the vehicle, Ad and Ad-HO-1 groups were respectively injected with 20 μl saline, saline containing 1.25 μl empty vector adenovirus or saline containing 1 μl recombinant HO-1 adenovirus via the right lateral ventricle once daily for 3 days prior to cerebral ischemia-reperfusion injury induction. The injection site was referred to as the Paxinos and Watson rat brain stereotactic atlas (10).

Transient focal cerebral ischemia-reperfusion injury models. Rats in the vehicle group, Ad group and Ad-HO-1 group were anesthetized (3% isoflurane for induction and 1% for maintenance) and the transient focal cerebral ischemia-reperfusion injury models were induced via the MCAO method and intraluminal suture technique (22,23). The rats in the sham group did not receive MACO or intraluminal suture, however a sham operation was performed. Blood flow was reduced to 87-90% in the ipsilateral parietal cortex, which was considered as confirmation of successful occlusion. During the experiment, a laser-Doppler flowmeter (Probe 407-1; Perimed AB, Järfälla, Sweden) was used for all groups to monitor the blood flow at a maintained body temperature (37.0±0.5°C). Following occlusion for 90 min, blood flow was restored for 90 min to the ischemic region of brain and the filament was removed from lumen. All rats recovered from anesthesia prior to being returned to their cages.

Detection of GFP. Following a 24-h recovery period from MCAO, rat MCAO models were anesthetized terminally with sodium pentobarbital (60 mg/kg; intraperitoneal; Sigma-Aldrich; Merck KGaA) and saline was used for transcardial perfusion. Rat brains were harvested, and sliced into 5 coronal sections (2-mm thick) from the frontal pole, fixed in 4% formalin overnight at 30°C and stained with 0.5% w/v triphenyltetrazolium chloride (TTC) dissolved in PBS for 15 min at room temperature. Infarct areas of each slice were analyzed using the SigmaScan Pro 5 Image Analysis software (Systat Software, Inc., Point Richmond, CA, USA). Direct GFP signals were digitally photographed using an Olympus BX51 fluorescence microscope (magnification, x200; Olympus Corporation, Tokyo, Japan). A total of 5 fields of view were randomly selected in each slice, 100 cells were randomly selected in each, and the number of fluorescent cells were counted. Subsequently, the efficiency of transfection was calculated as follows: Efficiency of transfection=number of positively stained cells/total number of cells x100%, which was automatically calculated and exported to Image-ProPlus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) for further analysis.

Western blot analysis. Total protein was extracted from cortices of SD rats in all groups using a radioimmunoprecipitation lysis buffer (Thermo Fisher Scientific, Inc.). Protein concentration was determined using the BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Equal amounts of protein (50 μg/lane) were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The western blot assays were performed with a Mini-PROTEAN®TGX™ system (Bio-Rad Laboratories, Inc., Hercules, CA, US). A 5% skimmed milk powder in Tris-buffered saline with Tween-20 blocking solution was used for 1 h at room temperature. The membranes were incubated with β-actin (cat. no. RB-9010-R7; 1:4,000; Invitrogen; Thermo Fisher Scientific, Inc.) and anti-HO-1 (cat. no. sc-120745; 1:3,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies overnight at 4°C. The membranes were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibodies (cat. no. 7076; 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 1 h. The protein bands were detected using the enhanced chemiluminescent method with ECL™ Western Blotting Detection reagents (GE Healthcare, Chicago, IL, USA) and subsequent autoradiography. The protein levels of the bands were normalized against β-actin.

Assessment of neurological deficit score (NDS). Following a 24-h recovery period from MCAO, the NDSs of rats were examined as described previously (18) with a modified scoring system. The modified scoring system was adopted to determine the neurological functional deficit at 4 h after the MCAO. Neurological deficit score (NDS; 0-10% normal, 100% maximum deficit). The modified neurological severity score (mNSS), a composite of motor, sensory, reflex and balance tests was used to test the sensorimotor deficit. The mNSS was graded on a scale of 0-18. A normal score was 0 and the maximal deficit score was 18, 1 point was awarded for the inability to perform the task or the lack of a tested reflex. (0, no neurological deficits; 1, failure to extend right forepaw fully; 2, circling to right; 3, falling to right; 4, did not walk spontaneously and has depressed levels of consciousness).

Volume of infarct regions. At 24 h following MCAO establishment, the tissues of rat brains were sectioned as described above. Six samples from each group were used in each experiment. The volume of infarct regions was assessed using TTC staining in the dark overnight at 30°C, and images of each section were captured using an Olympus BX51 fluorescence microscope (magnification, x200). The infarct areas of each slice were analyzed using the SigmaScan™ Pro V5.0 Image Analysis software (Systat Software, Inc., San Jose, CA, USA). The injured tissues remained unstained by TTC, as staining did not occur in areas where neuronal loss was observed. Therefore, the unstained areas (infarct tissues) were examined and analyzed with Photoshop software 7.0 (Adobe Systems, Inc., San Jose, CA, USA). The sections were used to determine the volumes of infarct region. For the ipsilateral hemisphere, the infarct regions of each brain were calculated in mm^3, which was corrected for swelling in the same brain with translation on the percentage of contralateral hemisphere as described previously (24).

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end (TUNEL) staining. At 24 h following MCAO, brain tissue sections were fixed in 4% formalin overnight at 30°C then stained with TUNEL (Roche Diagnostics GmbH; Mannheim, Germany) at 37°C for 1 h. The incorporated digoxigenin-conjugated nucleotides were detected using a horseradish peroxidase-conjugated anti-digoxigenin antibodies and 3,3'-diaminobenzidine. The dehydrated sections were cleared in xylene, mounted with Canada balsam and enclosed with coverslips. The sections of brain tissues (n=6 per group) in contralateral hemisphere and five fields of view were randomly selected for counting and NDSs were determined. Subsequently, brain tissue sections were dewaxed and rehydrated at 60°C for 2 h. These slides were incubated for 30 min at room temperature in a 20 μg/ml DNase-free proteinase K and rat terminal deoxynucleotidyl-transferase (both Sigma-Aldrich; Merck KGaA) reaction mix. Following rinsing with PBS 3 times for 15 min
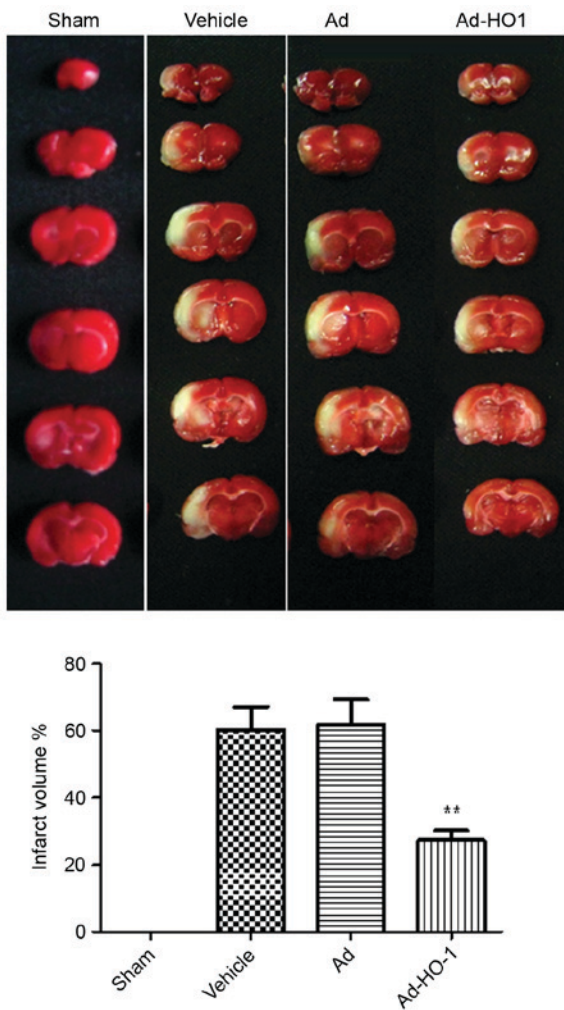
Figure 1. Overexpression of HO-1 attenuated infarct volume in the ipsilateral cortex following MCAO. Just after 2 h of MCAO, the blood flow was decreased to 80-90% of the baseline level in the core area, which classified the model as successful. Following 24 h of MCAO, the cerebral blood flow was not significantly different between the two model groups (vehicle and Ad groups). The total volume of infarction and infarction area of representative brain sections in each group were indicated at 24 h following ischemia. Compared with the sham group, volume of cerebral infarction in V and Ad groups were significantly increased. Over-expression of HO-1significantly attenuated infarct volume in the ipsilateral cortex. Data are presented as the mean ± standard deviation (repeats, n=6).**P<0.01 vs. vehicle and Ad groups. MCAO, middle cerebral artery occlusion; Ad, empty adenovirus vector; HO-1, heme oxygenase-1; Ad-HO-1, recombinant HO-1 adenovirus.

Figure 2. Expression of green fluorescent protein. No green fluorescent protein was detected in the (A) sham and (B) vehicle groups. However, significant expression of green fluorescent protein (transfection rate was 34.5±3.4%) was indicated in the (C) Ad (36.31±2.21) and (D) Ad-HO-1 groups (27.31±1.38) (P<0.01) at 24 h after ischemia-reperfusion injury was established (magnification, x400). (E) Quantification of fluorescence. **P<0.01 vs. vehicle and Ad groups. Ad, empty adenovirus vector group; HO-1, heme oxygenase-1; Ad-HO-1, recombinant HO-1 adenovirus.

each time, these sections were treated with streptavidin horseradish peroxidase (1:200; Roche Diagnostics GmbH) at 37˚C for 30 min and positive signals were enhanced with diaminobenzidine (1:200; Roche Diagnostics GmbH) at room temperature for 30 min. The tissue slide was digitally photographed using an Olympus BX51 fluorescence microscope (magnification, x400). The apoptotic index (positively stained cells count/total number of cells x100%) was automatically calculated and exported to GraphPad Prism 5.0 for further analysis.

Activation of caspase-3. The neuronal activity of caspase-3 was examined using a Caspase-3 Activity Assay kit (cat. no. CST 5723S; Cell Signaling Technology Inc.) according to the manufacturer's protocol. Brain tissues of the total suspension weighing 3 g were homogenized for 2 min in Mcllwain's buffer,

then the homogenates were centrifuged at 3,000 g for 5 min at room temperature and the supernatants were collected.

Statistical analysis. The normally distributed continuous variables were indicated as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 5.0. Between groups, the Student's t-test (for two group comparisons) or one-way analysis of variance with Kruskal-Wallis post hoc tests (for multiple group comparisons) were used to analyze and compare quantitative data. The data that were abnormally distributed were analyzed between groups with Kruskal-Wallis analysis of variance method. SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA) was used to analyze these statistical data. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of HO-1 protects against transient cerebral ischemia-reperfusion injury. MCAO establishment was considered successful when the blood flow of the rat models was decreased to 80-90% of the baseline level in the core area. Following MCAO establishment, the cerebral blood flow was

Figure 4. TUNEL stain. In the brain tissues of the sham group, the percentage of positive cells stained with TUNEL was 0.1% (magnification, x400). Conversely, in the tissues from brains of ischemic-reperfused rats, TUNEL-positive nuclei were prevalent. However, the number of TUNEL-positive cells was markedly reduced following treatment with Ad-HO-1. (A) Sham group, (B) Vehicle group, (C) Ad group and (D) Ad-HO-1 transfection group. (E) The results were quantified. ***P<0.001 vs. the vehicle group and ###P<0.001 vs. the Ad group. TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end staining, Ad, empty adenovirus vector; HO-1, heme oxygenase-1; Ad-HO-1, recombinant HO-1 adenovirus.

treatment with HO-1 gene (18.83±1.82) compared with the Ad group (59.33±2.95; P<0.001) (Fig. 4). These findings suggest that HO-1 gene overexpression may have an inhibitive role in post-ischemic neuronal apoptosis.

Overexpression of HO-1 with recombinant adenovirus reverses behavioral impairments induced by secondary brain damage in transient cerebral ischemia-reperfusion injury. Transient cerebral ischemia-reperfusion damage markedly reduced the NDS (Fig. 5) at 24 h following MCAO. The neurological deficit scores were significantly reduced in the MCAO groups (Vehicle and Ad group) compared with the sham group (P<0.01). Overexpression of HO-1 significantly ameliorated the NDS (12.45±0.18%) compared with the vehicle (9.82±0.17%) and Ad (9.96±0.09%) groups (P<0.001; Fig. 5). These findings suggest that overexpression of the HO-1 gene has a potential protective role against secondary brain damage of the cerebral cortex induced by
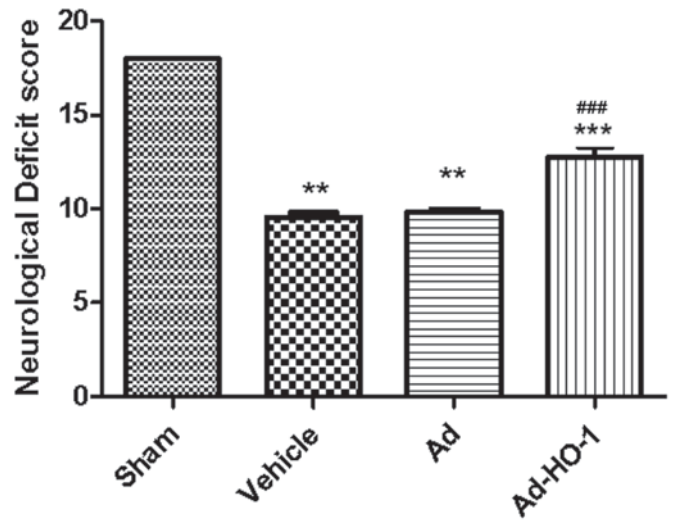
Figure 5. Overexpression of HO-1 via recombinant adenovirus transfection reversed behavioral impairments induced by secondary brain damage in transient cerebral ischemia-reperfusion injury. Transient cerebral ischemia-reperfusion damage reduced the neurological score at 24 h following middle cerebral artery occlusion. Overexpression of HO-1 significantly attenuated the neurological deficit score compared with transient cerebral ischemia injury groups (vehicle and Ad groups). Data are presented as the mean ± standard deviation (repeats, n=6). **P<0.01 vs. the sham group; ***P<0.001 vs. vehicle; ###P<0.001 vs. the Ad group. Ad, empty adenovirus vector; HO-1, heme oxygenase-1; Ad-HO-1, recombinant HO-1 adenovirus.

transient cerebral ischemia-reperfusion injury, and may restore neurological/behavioral impairment.

Discussion

Globally, stroke is one of the major causes of long-term disability and death (25). Stroke is associated with notable socioeconomic and clinical implications; furthermore, there is an urgent requirement for effective therapies to treat stroke. In practice, stroke is a pathological process involving multiple factors that are typically associated with oxidative stress, inflammation, calcium overload, brain edema and cell apoptosis (26).

TIAs (transient ischemic attacks) are known as brief ischemic episodes and have been studied for over two decades (27,28). Tolerance to stroke may be induced by TIAs through elevating the safety threshold of human brain tissue (29). The potential molecular mechanisms of neuroprotection induced by TIAs were unclear (29). No clinically effective therapies are currently available for treating stroke, despite a number of studies based on experimental neuroprotective compounds and animal models that have demonstrated promising results (30,31). Investigation into the potential molecular mechanisms associated with TIAs may aid the identification of the novel targets for the prevention and treatment for stroke (32).

HO-1 has a crucial role in the normal function of neurons (33). In normal rats, the expression of HO-1 is located at the neuronal nuclei of Purkinje or hindbrain cells in the cerebellum (34). Furthermore, heat shock proteins, trauma, bleeding and hypoxia may induce the expression of HO-1 (35). HO broadly participates in the functional regulation of the heart, brain, lungs, liver, kidneys and other tissue

cells associated with anti-oxidative stress injury (36). Previous results have demonstrated that the increase in HO expression was able to protect neuronal cells against ischemia through anti-oxidative stress (37), anti-apoptosis (38) and inhibition of the inflammatory response (39), thereby improving the cerebral microcirculation blood flow, stabilizing the cell membrane and mitochondrial membrane, and reducing brain edema (40). IPC induces the expression of HO-1, which has an important role in mediating the protection against transient and permanent brain ischemia (41). Additionally, following permanent ischemia, HO-1 may be the critical factor in improving the NDS and reducing infarct volume of the brain (42). In summary, HO-1 is the key element in the protection against stroke, particularly in the tolerance to stroke induced by IPC (3).

Conventional therapeutic strategies, which are administered via a range of methods, including orally, subcutaneously, intramuscularly and intravenously, are subject to different degrees of blood-brain barrier obstacles, which makes the outcome of an effective therapeutic response a challenge. As a novel therapeutic strategy, brain stereotactic injection of exogenous gene therapy for brain disease has received a lot of interest in recent years (43).

In the present study, the recombinant adenovirus vector of HO-1 was transfected into rats on the right lateral ventricle and the HO-1 gene was injected directly into the brain. The protein expression levels of HO-1 were continuously detected for 3 days following transfection, and were significantly increased in the cortex compared with the Ad group. However, protein expression levels of HO-1 were decreased in the Ad group transfected with adenovirus vector compared with the Ad-HO-1 group, which demonstrated that the injection method of intracerebroventricular administration was feasible and effective.

The present results indicated that intraventricular injection with Ad-HO-1 is an effective method to enhance the expression of HO-1 and reverse brain injury induced by transient focal cerebral ischemia-reperfusion injury in rats. Furthermore, the present findings suggested that this approach may significantly inhibit neuronal apoptosis and attenuate neurological behavioral deficits. The results also demonstrated that rats that received MCAO and pretreatment with HO-1 gene exhibited a significant decrease in infarct volume compared with rats that were subjected to MCAO and treated with vehicle or Ad. Furthermore, the present findings indicated that intraventricular injection with the HO-1 gene significantly induced the expression of HO-1, which improved neurological behavioral. This intervention method may lead notable clinical benefits through decreasing the volume of infarct at a later time point (at 24 h after reperfusion).

Similar to reports in previous studies (44,45), the present study demonstrated that the expression of HO-1 increased significantly in focal ischemic brains, which indicated that HO-1 may be a potential sensor for therapy *in vitro*. In the present study, a common adenovirus vector was used for the control, which did not affect the expression level of HO-1. However, in the Ad-HO-1 group, expression was significantly increased. These results demonstrated that the protective effects originated from the successful expression of HO-1 introduced by transfection compared with endogenous HO-1.

In conclusion, the HO-1 gene carried by adenovirus may be effectively transfected into the brain tissue and expressed stably *in vivo*. The programmed cell death in neuronal cells following cerebral ischemia-reperfusion injury may be significantly reversed by overexpression of HO-1. The results of the present study suggested that HO-1 was able to mediate the effective reduction of infarct volume, and provide neuroprotection against transient cerebral ischemia-reperfusion induced brain damage.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL performed all experiments analyzed and interpreted the data. RG, WH and ZS performed some of the experiments. GW, LW and YX were major contributors in writing the manuscript and contributed to the conception and design of the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by The Second Hospital of Shanxi Medical University Committee on Animal Care (Shanxi, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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